

Quantification of lipid in cultured 3T3-L1 adipocytes

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Abstract

A preliminary study was conducted to quantify the lipid produced by differentiated 3T3-L1 cells after incubation in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS), supplemented with or without dimethyl-sulphoxide (DMSO; 9.6 g/l) and acetone (1.2 g/l). The two media treatments were applied to 3T3-L1 cells, plated at either 15K or 30K cells per well in 24-well plates. Cells were grown to confluence (96 h) and then treated with dexamethasone, methyl-isobutylxanthine and insulin for 48 h and later maintained in their respective media treatments for another 144 h. Cells from each treatment were recovered after two, 5-min incubations with trypsin, washed and resuspended in DMEM and counted on a haemocytometer. The lipid in the cells was extracted with hexane derivatized with tetramethyl-guanidine and analysed by gas chromatography. Final mean cell density was 6.8 (s.e. 0.18) $\times 10^5$ and 4.6 (s.e. 0.19) $\times 10^5$ when initially plated at 30K and 15K cells per well, respectively. Inclusion of DMSO and acetone in the medium did not affect final cell numbers. Plating density did not affect concentration of lipid (0.55 (s.e. 0.08) mg per 1 $\times 10^5$ cells) but inclusion of DMSO and acetone led to overall decreases in total lipid concentration. Results indicate that initial plating density influenced final cell number in treatment cultures, but that DMSO and acetone treatments only had an effect on final lipid concentration. Collectively, these data suggest that the application of treatments to cell cultures may be influenced by the carrier vehicle that the treatment is contained in and this should be considered when developing an in vitro system to evaluate growth and development of adipocytes.

Keywords: adipocytes, gas chromatography, haemocytometer, plating density, lipids.

Introduction

There are many indications that adipose depots from different locations in animals, especially cattle, exhibit different characteristics. Isozymes of diacylglycerol acyl transferase expressed at subcutaneous and intra-muscular sites were found to have different requirements for cofactor and pH optima (Middleton *et al.*, 1998). Substrate specificities were acetate and glucose for triglyceride synthesis at these two sites respectively (Smith and Crouse, 1984). These differences in enzyme preferences at the two adipose sites indicate a need to study specifics of adipose development in isolation. In order to conduct investigations to determine factors that affect development of bovine adipocytes, it was necessary to collect information about the impact of cell density and of solvents used to deliver compounds on different aspects of adipocyte development. Thus these preliminary investigations were undertaken using 3T3-L1 cells.

The pre-adipocyte cell line 3T3-L1 is widely used to study numerous aspects of lipid metabolism of mammalian adipocytes, including the effect of compounds (e.g. hormones, fatty acids) on cell differentiation (Sadowski *et al.*, 1992; Satory and Smith, 1999) and lipid accumulation in the cells of adipose tissues. Cell differentiation is studied by measuring the activity of many enzymes that participate in lipid accumulation (Sadowski *et al.*, 1992; Suryawan *et al.*, 1997). However, lipid accumulation has been measured by counting oil red-O stained cells, and enzyme activity is relied upon for estimating lipid synthetic activity. Although these techniques are widely accepted, they do not provide an indication of the amount of lipid produced, and when cell densities are high, counting cells is difficult. Situations are possible where there may be numerous adipocytes with little fat and few adipocytes with large quantities of fat. Therefore, a method for determining the amount of fat produced

is necessary. It was thus postulated that direct measurement of accumulated lipid would be possible if the lipid from differentiated adipocytes could be extracted and analysed for fat content as total fatty acids by gas chromatography.

The effect of many compounds (e.g. retinoic acid) on lipid accumulation is determined by applying them in fat and water-miscible compounds, such as dimethyl sulphoxide (DMSO) and ethanol (Ohya *et al.*, 1998) in cell culture assays. The effect of these carrier compounds on the different aspects of lipid accumulation in adipocytes is not known. Satory and Smith (1999) have studied inclusion of conjugated linoleic acid on adipocyte function but the levels used in their study are at a maximum of 10 mg/l. This concentration is not representative of concentrations that would be reached if consumption levels reached recommended amounts and larger concentrations of ethanol would be required to maintain the fatty acids in solution, which could be toxic to cells. Therefore alternative, less toxic, solvents needed investigation. This project was conducted to determine the effect of the inclusion of DMSO and acetone in future experiments to deliver water insoluble compounds (e.g. fatty acids) in media to adipocytes. The effect on total cell number and lipid content was determined in cultures of 3T3-L1 cells, plated at 15 and 30 K ($K = 10^3$) densities, by counting treated cells in a haemocytometer after lifting them by trypsin treatment. The fat content in the cells was determined by conducting gas chromatography on the lipid extracted from the cultures. Lipid accumulation in cells was visually inspected after staining as well.

Material and methods

3T3-L1 cells were thawed, washed free of DMSO (from the freezing medium) with Dulbecco's Modified Eagle Medium (DMEM) containing 100 ml/l foetal bovine serum (FBS), plated in a 150-mm culture dish and incubated for 24 h in an incubator with 0.05 carbon dioxide. The cells were maintained for 2 days with media changes every 24 h. When cells were at peak growth, they were rinsed with phosphate buffered saline (PBS, pH 7.08) and incubated at 37°C for 5 min with 10 ml of PBS containing 100 g/l ethylene diamine tetra-acetic acid (EDTA) and trypsin (2.5 g/l). Cells were transferred into a sterile tube with an equal volume of DMEM containing 100 ml/l FBS. The cells were centrifuged at 1000 r.p.m. for 5 min and the supernatant discarded. Cells were re-suspended in 10 ml DMEM containing 100ml/l FBS and counted on a haemocytometer. The number of cells required for the experiment was left in the tube and the remaining cells were refrozen. The cells in the centrifuge tube

were diluted with DMEM plus 100 ml/l FBS such that cell density was 30×10^3 cells per ml. The cells were plated at 30×10^3 cells per well, in eight replicates in experiment one and in 16 replicates in experiment two, in 24-well plates. The remaining cells were diluted with an equal volume of DMEM containing 100 ml/l FBS to yield cell densities of 15×10^3 cells per ml, and eight wells were plated in experiment 1 and 16 wells were plated in experiment 2. Four or eight wells, in experiment 1 or 2 respectively, plated at each cell density were continued with DMEM containing 100 ml/l FBS, while the other four or eight wells in experiment 1 or 2 respectively, were provided with the same medium but which also contained 9.6 g/l DMSO and 1.2 g/l acetone (ACE). Media in all wells were changed every 24 h.

At 96 h after the introduction of treatments, the cells in all wells were provided with their respective medium, which also contained 0.1 µmol/l dexamethasone, 1mmol/l methyl-isobutylxanthine and 0.1 µmol/l insulin (DMI) for 48 h (Vierck *et al.*, 1996). After 48 h, media without DMI was provided for another 144 h with media changes every 24 h. At the end of the incubation period, the cells in three wells in experiment 1 and seven wells in experiment 2, at each plating density, within each media treatment were recovered into individual test tubes. To recover the cells, wells were first washed with PBS, incubated with PBS containing 100 g/l EDTA and trypsin (2.5 g/l) for 5 min and then transferred to centrifuge tubes. The trypsin treatment of the wells was repeated twice to ensure complete recovery of the cells. Trypsin was inactivated by the addition of an equal volume of medium containing 100 ml/l FBS. The cells recovered from each well were counted in a haemocytometer to obtain the total number of cells in each well and then washed with PBS. The cells were further extracted with four, 1.0 ml aliquots of hexane. The hexane layer was quantitatively transferred to another tube and the hexane was vaporized under a stream of nitrogen to collect the lipid produced by the cells. The lipid was methylated with tetramethylguanidine and analysed by gas chromatography for fatty acids (Mir *et al.*, 1999). The fatty acids measured were C14 : 0, C16 : 0, C16 : 1, C18 : 0, C18 : 1, C18 : 2 and C18 : 3. The areas for fatty acid peaks were summed to obtain total fatty acids and represented fat in the cells from the wells plated at the two densities and treated with or without DMSO and ACE and expressed as total lipid per 10^5 cells. Remaining wells in both experiments for both media treatments, with or without DMSO and ACE, were stained with oil red-O and counter stained with giemsa stain for visualization of lipid in the cells. The effect of media treatment and plating density on ultimate cell number and lipid content was

Table 1 Total cell count and lipid concentration in wells of 3T3-L1 cells plated at different densities†

	30 × 10 ³ cells per well				15 × 10 ³ cells per well			
	DMEM + FBS		DMEM + FBS + DMSO + ACE		DMEM + FBS		DMEM + FBS + DMSO + ACE	
	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
Total cell count (× 10 ⁵)								
Experiment								
1	6.98 ^a	0.18	6.65 ^a	0.35	4.43 ^b	0.08	4.68 ^b	0.43
2	5.90 ^a	0.26	4.94 ^a	0.21	3.90 ^b	0.25	3.20 ^b	0.28
Lipid concentration (mg/10 ⁵ cells)								
Experiment								
1	0.54 ^a	0.002	0.10 ^b	0.002	0.57 ^a	0.06	0.08 ^b	0.05
2	0.009 ^a	0.003	0.003 ^b	0.001	0.010 ^a	0.003	0.001 ^b	0.001

^{a,b} Means in a row not sharing common superscripts differ ($P < 0.05$).

† Treatment codes: DMEM = Dulbecco's Modified Eagle Medium; FBS = foetal bovine serum; DMSO = dimethyl sulphoxide; ACE = acetone. No. = 3, in experiment 1 and 7, in experiment 2.

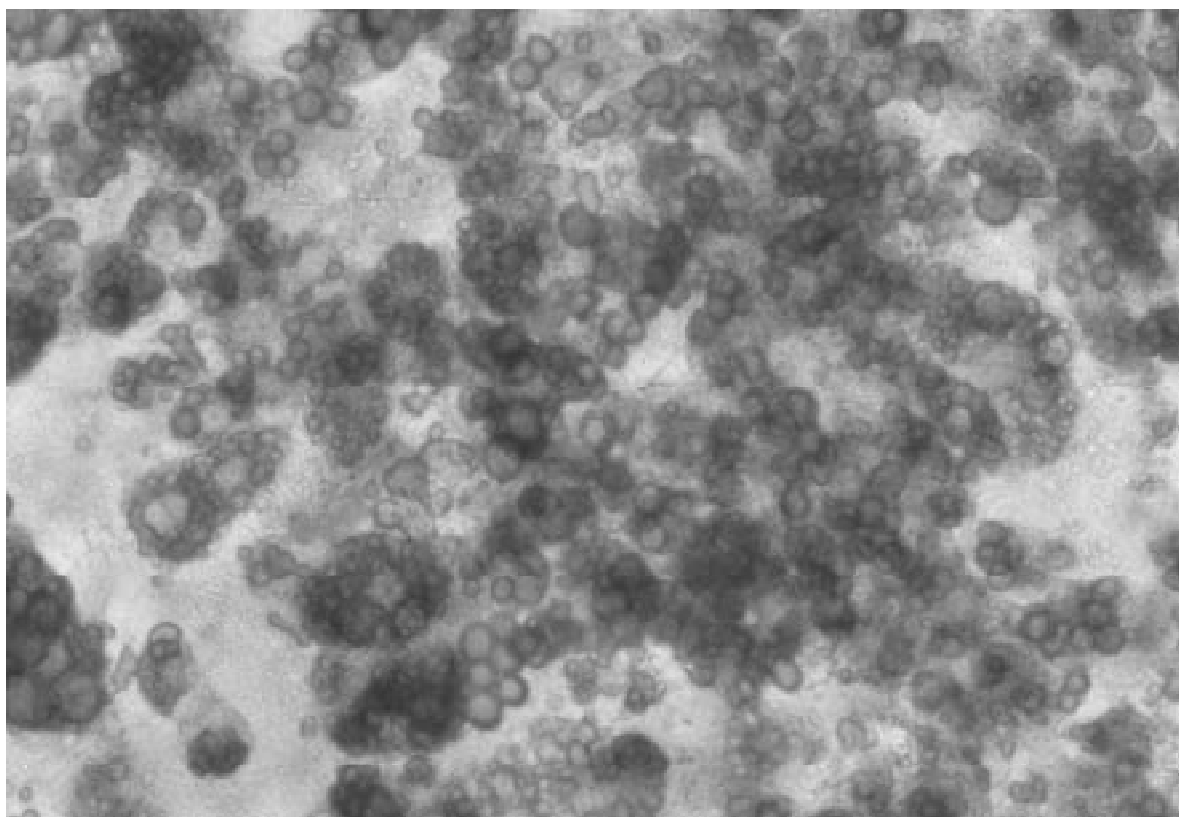


Figure 1 3T3-L1 cells plated at 30 K per well and treated with DMEM and 10% FBS and 48 h of DMI (40 × magnification, slide no. 28).

analysed by conducting analyses of variance and differences among means was determined by the protected least significant difference.

Results and discussion

In both experiments plating densities of 15×10^3 cells per well resulted in lower numbers of cells per well, ultimately, than when plated at a density of 30×10^3 cells per well (Table 1). Inclusion of water and oil miscible solvents in the media did not affect cell proliferation and differentiation, as cell numbers were not affected ($P > 0.05$) and lipid accumulation could be visualized in wells treated with either medium (Figures 1 and 2). Plating density did not affect lipid accumulation in cells since concentration per 10^5 cells was similar for both plating densities. However, the inclusion of water and oil miscible solvents such as DMSO and acetone decreased lipid concentration substantially at both densities (Table 1) and can be seen as smaller oil bodies in cells treated with the solvents (Figures 1 and 2).

Although Ohyama *et al.* (1998) and Satory and Smith (1999) have used ethanol as a vehicle for delivery of their compounds (retinoc acid and conjugated linoleic acid or linoleic acid, respectively) to adipocytes, an assessment of the effect of the vehicle is not included in their reports. Satory and Smith (1999) used a maximum concentration of 10 mg/l of both the conjugated linoleic acid and linoleic acid in their study. If fatty acid concentrations used in *in vitro* systems were to approximate levels attainable in *in vivo* studies, the concentrations would have to exceed 37 mg/l (Mir *et al.*, 2000) and might require greater concentrations of ethanol, than the 1.0 ml/l used by Satory and Smith (1999), to maintain the fatty acids in solution. The need to determine effect of higher concentrations of the fatty acids is related to the recommended intake of conjugated linoleic acid (CLA) to obtain adequate chemo-protection against cancer (Ip, 1997), which is 1% of diet and is estimated to be 3.5 g/day. If we assume complete absorption of the fatty acid and distribution in all body fluids, which can be assumed to be

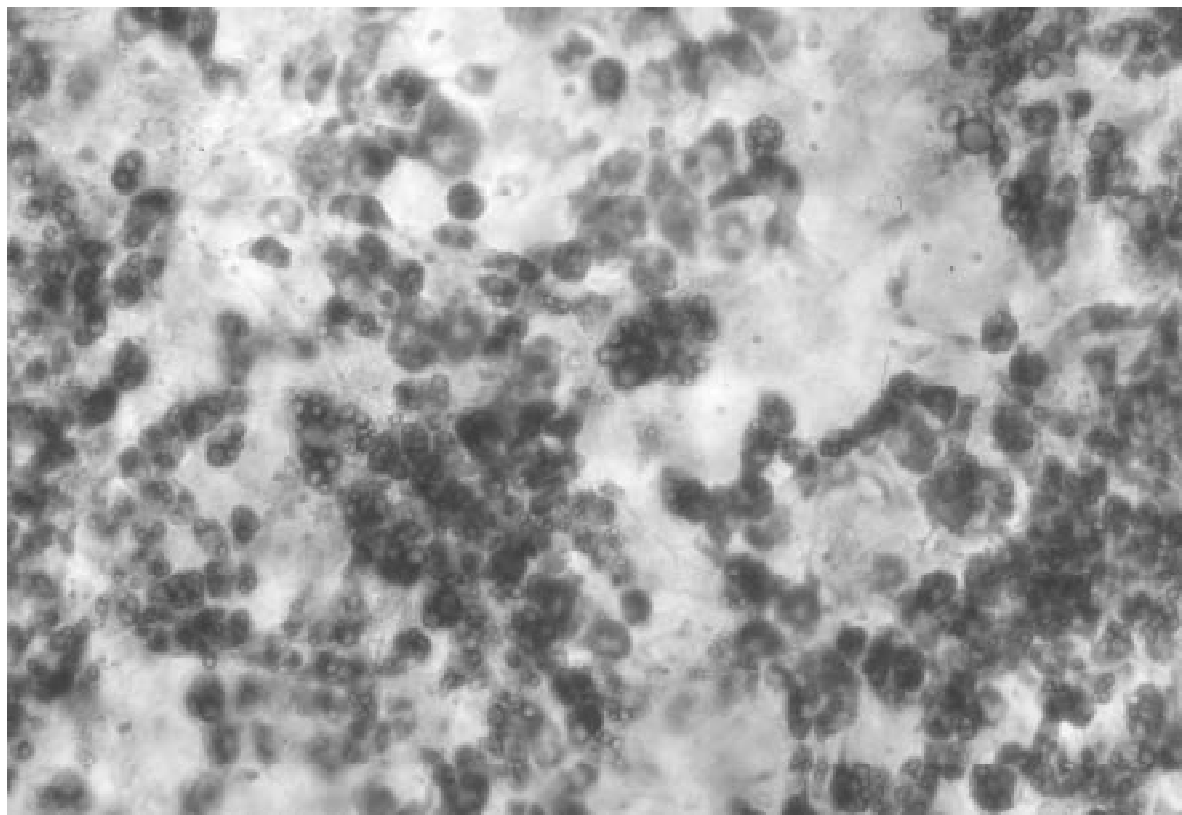


Figure 2 3T3-L1 cells plated at 30 K per well and treated with DMEM and 10% FBS, DMSO and ACE and 48 h of DMI (40 \times magnification, slide no. 26).

proportionately 0.7 of body weight, then in a 70-kg reference man the concentration would be 71 mg/l. This concentration is greater than the highest concentration (10mg/litre) studied by Satory and Smith (1999). In a previous experiment Mir *et al.* (2000) offered lambs that weighed 12.5 kg, 330 mg of CLA daily and if we were to calculate a dilution from that feeding rate, with the expectation that all of the CLA would be absorbed, a concentration of 37 mg/l would be required in culture media. In order to achieve such high concentrations of the individual fatty acids it might be necessary to include higher concentration of ethanol than that used by Satory and Smith (1999), which might be toxic to the cells. As a result we needed to explore other solvent systems and their effects on adipocyte function needed evaluation.

Commonly, enzyme assays are used to measure the effect of treatment on lipid production (Satory and Smith 1999). Only Satory and Smith (1999) have used gas chromatographic analysis to measure lipid production from 3T3-L1 adipocytes, but this was conducted in a slightly different manner from in the present study. Satory and Smith (1999) counted aliquots of each sample after treatment with trypan blue to assess viability of cells, while cell counts post induction were estimated by thymidine incorporation in parallel wells, which were separate from those used for lipid extraction. In the present study the cells were retrieved from the wells, counted in a haemocytometer and the lipid content of the same cells was measured by gas chromatography after extraction with hexane. Satory and Smith (1999) used the Folch extraction procedure, which extracts substantial amounts of membrane lipids quite unlike hexane, which is specific to neutral and storage lipids. The composition of the fat can also be determined by this technique. In the present study the principal fatty acids were palmitic acid (C16:0) and palmitoleic (C16:1) acid, which is as expected because the fatty acid formed by *de novo* synthesis is largely C16:0 and these results are similar to those previously described (Satory and Smith, 1999).

These data suggest that the application of treatments to cell cultures can be influenced by the carrier vehicle for the treatment and should be considered if developing *in vitro* systems to evaluate growth and development of adipocytes. The absence of differences in cell numbers between those treated and not treated with the solvent system (DMSO and ACE) indicates that proliferation was not affected by the presence of the solvents in the media. Oil red-O staining of cultured adipocytes treated with either medium indicated that differentiation was observed

in both culture systems. However, lipid accumulation was lower in cells treated with the medium containing the solvent system. This was observed in both experiments. Although, the cause of the decrease in lipid accumulation is not known it is possible that the solvents extracted formed lipids whenever the media were changed. Visual inspection of stained wells at the end of the experiment did not reveal evidence of ruptured cells due to treatment.

Although, the effect of plating density and media treatment on lipid content was similar in both experiments, the amount of lipid in the second experiment was substantially lower. The reason for this difference is unknown but a lower level of differentiation was observed for all wells in this study. The stained lipid droplets were smaller than those observed in the first experiment and it is suspected that this affected the overall lipid content of the cells.

Data from the present investigation indicate that cell number and total lipid accumulation can be measured by using a haemocytometer to count the cells, even when densities are high. The amount of lipid in cells can be determined by gas chromatography to obtain an assessment of lipid produced. These preliminary studies were required to establish the parameters under which future work can be conducted to determine the relative influence of effector compounds on various activities of mammalian adipocytes from different locations in the animal.

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